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Review

Chitosan and its derivatives—a promising non-viral vector for gene transfection

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Abstract

The ability of a vector to ferry gene into targeted cells is the premise of improving gene-transfer efficiency. Chitosan, a naturally occurring cationic polysaccharide, has been shown to excel in transcellular transport. This attribute has been fully reflected in chitosan-mediated gene transfection systems. The objective of this review is to summarize the recent encouraging advances in unveiling the mechanism of cell entry and application of chitosan and its derivatives as novel non-viral vectors. It is our belief that researchers will uncover more truth about chitosan-based vector and realize the long-term goal of gene transfection—produce the desired clinical effect.

Keywords: Chitosan; Gene transfection; Non-viral vector

1. Introduction

Gene therapy refers to the transmission of DNA encoding a therapeutic gene of interest into the targeted cells or organs with consequent expression of the transgene. In the past several years, gene therapy has received significant attention due to its potential application in the replacement of dysfunctional gene and treatment of acquired diseases [1,2]. The central problem of gene therapy lies in the development of safe and efficient gene transfection system. Although nakedDNA has been found to transfect some kinds of cells like the skeletal muscle cells of the cardiac and the diaphragm region, its electronegativity tends to inhibit itself from entering most negatively charged cell membranes. Moreover, the unprotected DNA is rapidly degraded by nucleases present in plasma. Recently many techniques have been developed for the introduction of DNA into cells. Systems currently under study for in vitro and in vivo use include both viral and non-viral vectors. Virus vectors are very effective in terms of transfection efficiency, but they have fatal drawbacks such as immune response and oncogenic effects when used in vivo. Gelsinger's death from a gene therapy clinical trial in 1999 prompted a hard look at the safety record of the viral vectors, and spurred a renewed interest in non-viral methods to ferry genes into tissue [3]. Among the non-viral vectors currently investigated, polyelectrolyte complexes (PEC) between DNA and polycations have been extensively investigated. DNA is tightly packed in the PEC complex, so that the entrapped DNA is shielded from

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contact with DNAses [4–6]. The cationic polymers include polyethyleneimine [7], poly(L-lysine) [8], dendrimers [9], polybrene [10], gelatin [11], tetraminofullerene [12], poly(L-histidine)-graft-poly(L-lysine) [13]. Although PEC systems have some advantages over virus vector, e.g. low immunogenicity and easy manufacture [14,15], several problems such as toxicity, lack of biodegradability, low biocompatibility and in particular, low transfection efficiency need to be solved prior to practical use [16]. In elucidating the biological barrier to cellular delivery of lipid-based DNA carrier, Bally et al. [17] outlined six steps and associated obstacles involved in gene transfection (Fig. 1).

As shown in the diagram, to realize the ultimate clinical therapeutic effect, the exogenous gene must successfully cross multiple-blockages. A spate of recent work has suggested that DNA wrapped in inter-polyelectrolyte complexes is well protected from DNase degradation. To overcome the second barrier, the PECs are constructed so that the surface of structure charge exhibits a net charge. It is widely accepted that the positive charge facilitates binding to cell membrane, which is not surprising since cell membrane is negatively charged. But Bally argues that this explanation is too simplistic. The complex particles tend to aggregate in the presence of salts and the aggregation reaction leads to large particles to sediment, thereby facilitating cell contact, particularly in cultures of adherent cells. Similar results have been reported by Eastman and Nakanishi et al. [18,19].

The ability of a vector to transport gene into targeted cells is the premise of improving gene-transfer efficiency. But the mechanism of gene transfer across the cell membrane is not well understood, and to date, the cationic polymers explored as

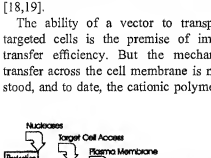


Fig. 1. The six steps and associated biological barriers involved in gene delivery.

non-viral vectors are relatively poor in carrying DNA molecules across membrane. In studying chitosan-based controlled release systems, researchers find that apart from the biocompatibility, biodegradability and low toxicity, chitosan excels in enhancing the transport of drugs across the cell membrane [20–22]. Most recently chitosan has been expanded to the field of gene transfection, and many encouraging results have been published. Considering its specific features in shuttling gene into cell, in this review we will focus on the recent achievements in studies of the mechanism of transcellular transport, the application of chitosan and its derivatives as non-viral vector.

2. Mechanism of transcellular transport

Chitosan was demonstrated to promote the nasal absorption of insulin in rats and sheep and to enhance the paracellular transport of peptides in vitro and in vivo by opening the tight junctions [23]. Holme et al. used the transepithelial electrical resistance (TEER) to investigate the effect of chitosan with various molecular weights and degrees of deacetylation on the permeability of human intestinal epithelial cell (Caco-2). It was found that chitosan with a high degree of deacetylation and degree of polymerization >50 induced the greatest effect on the opening of tight junctions of cells [24]. Chitosan has also been shown to bind mammalian and microbial cells by interacting with surface glycoproteins, and some studies have indicated that chitosan may actually be endocytosed into the cell. For example, chitosan microspheres were taken up by murine melanoma B16F10 cells via phagocytosis [25]. In repeated adhesion studies, it has been proven by Lehr et al. [23]that chitosan is fairly mucoadhesive in comparison to polycarbophile.

Chitosan consists of glucosamine units ((1,4)-linked poly(2-amino-2 deoxy-β-D-glucose or poly (D-glucosamine)) and has an apparent pK_a of 6.5. It aggregates in solutions at pH values above 6. It is therefore only soluble in acidic solutions (pH 1–6) where most of the amino groups are protonated. Recent studies have shown that only protonated soluble chitosan, i.e. in its uncoiled configuration can trigger the opening of the tight junctions, thereby

facilitating the paracellular transport of hydrophilic compounds [22]. This property implies that chitosan would be effective as an absorption enhancer only in a limited area of the intestinal lumen where the pH values are close to its pK_a (e.g. proximal duodenum).

Leong's group [26] investigated the permeation mechanism of chitosan across dipalmitoyl-*sn*-glycerol-3-phosphocholine-membrane bilayer. DSC was used to elucidate the thermotropic behavior of DPPC–chitosan mixtures. Cross-polarization microscopy was applied to determine the structural features of multilamellar vesicles (MLV). The effect of hydrophobic driving force on the chitosan–DPPC interaction was also addressed. They evidenced that chitosan induced the fusion of multilamellar vesicles, and the attractive interchain and intermolecular forces of the hydrophobic core (acyl chains) in the DPPC bilayer were significantly reduced by chitosan–membrane interactions. The addition of chitosan also reduced the order in the two dimensional packing of the acyl chains and increased the fluidity of DPPC bilayer. The study provided a valuable insight into the mechanism of chitosan-induced perturbation of a model membrane.

In contrast to its applications, the physiochemical properties of chitosan have not been extensively studied in the fields of colloidal and polymer sciences. Only recently has Berth et al. determined the radius of gyration of chitosan [27]. The study established the relationship between the molecular weight (M_w) and radius of gyration (R_g) of chitosan in aqueous solution, and further revealed that chitosan behaved more like a Gaussian coil instead of the worm-like chain model found in common polyelectrolytes. At low pH the primary amine along the backbone of chitosan is fully protonated. Therefore, the size of chitosan and pH are two important parameters that would dictate its permeabilizing and perturbating effects on the cell membrane. In their subsequent work, Leong's group investigated the effect of molecular weight and pH on the interactions of phospholipid bilayer with chitosan [28]. Reduction of pH increased the number of protonated amines on the chitosan backbone and caused further disruption of the membrane organization. It was found that the cooperative unit of chitosan was significantly reduced with the increase of chitosan mole fraction. At a chitosan mole fraction of 0.04%, the increase in molecular weight from 113 to 213 kDa resulted in a dramatic reduction of cooperative unit from 155 to 43. Berth et al. [27] showed that R_g values of 113 and 213 kDa chitosan were 47 and 54 nm, respectively, in aqueous solution. Once chitosan mole fraction was raised beyond 0.23%, the cooperative unit of 113 and 213 kDa chitosan reached a steady state of 52 and 24, respectively. The significant decrease of cooperative unit against chitosan molecular weight implied that chitosan swirled across the bilayer.

Chitosan becomes a polycation when its primary amines are protonated at pH equal to its pK_a. At the same time, the presence of *N*-acetyl groups on the chitosan backbone imparts hydrophobic properties. As a polyelectrolyte, chitosan tends to aggregate in aqueous solution, but few of studies toughed upon this behavior [29–31]. Recently, Philippova et al. [32] observed two types of hydrophobic aggregates in aqueous solutions of chitosan and its hydrophobically modified (HM) derivative. They proposed an aggregation model related to hydrophobic domains typical for different associating polymers with hydrophobic side chains and hydrophobic domains inherent to chitosan itself. In terms of the accumulated knowledge of physiochemical properties of chitosan, it is reasonable to consider that the high ionic strength and significant degree of acetylation of chitosan would result in a combined electrostatic-hydrophobic driving force for chitosan-induced destabilization of cell membranes.

In chitosan-mediated transfection, Venkatesh et al. [33] argued that in addition to ionic interactions, non-ionic interactions between the carbohydrate backbone of chitosan and cell surface proteins might have an important role in the chitosan-mediated transfection of cells.

3. Chitosan–DNA polyelectrolyte complex (PEC) systems

Mumper [34] was the first to propose to deliver gene into cell using chitosan as a vector. Chitosan–DNA complex with mean sizes ranging from 150 to 600 nm was formed by mixing a certain ratio of chitosan to plasmid DNA. The particle sizes were found to rely on the molecular weight of the chitosan

(108–540 kDa) used, but not on buffer composition. Other researchers also reported the effect of molecular weight of chitosan, DNA concentration, salt concentration, pH of media, charge ratio and temperature on the size of PEC. The influencing parameters have been reviewed by Janes [35] and Borchard [36]. From the drug delivery standpoint, the size of the particles is particularly important. Size variation will strongly affect the blood circulation time, entry into target cells and bioavailability of the particles within the body [37–42].

Objectively speaking, few of researchers have made efforts to correlate the transfection efficacy with the size of chitosan–DNA PEC, though there are several reports supporting the use of chitosan for gene transfection [11,43,44]. Typically, the particles smaller than 100 nm can be enclosed within endocytic vesicles, allowing entry into target cells via transferrin cytosis [45]. Whereas for DNA–chitosan PEC systems, several studies have shown that particle size ranges from 80 to 500 nm in 0.15 M NaCl [46]. Moreover, Erbacher et al. proposed that zeta potential was close to 0 mV ($N/P = 2$) with a size range of 1–5 μm [43]. In studying cationic cholesterol derivative-mediated gene transfection, Nikanishi [18] found that moderate size of particle (0.4–1.4 μm) yielded the highest transfection; while small vesicle less than 400 nm showed lower transfection efficiency. The results obtained by different authors seem to be contradictory. Thus, the mechanism of cationic polymer-mediated transfection is still underappreciated.

Recently, Sato investigated in detail the effect of pH, serum concentration, and molecular weight of chitosan on the transfection efficiency [47,48]. To determine in vitro gene transfer capability of chitosan, transfection of human-lung carcinoma A549 cells by luciferase plasmid (pGL3) was carried out in serum medium at different pHs. The transfection efficiencies at pH 6.9 are higher than that at pH 7.6. At below pH 7, amine moieties in chitosan are protonated, and the DNA–chitosan complexes are positively charged, which facilitates the binding with the negatively charged cells.

Fig. 2 shows the effect of molecular weight of chitosan on luciferase activities for A549 cells, B16 melanoma cells, and Hela cells. Chitosans of 15 and 52 kDa largely promote luciferase activity for all cell lines employed. Heptamer (1.3 kDa) does not show any transfection efficiency. Transfection efficiency mediated by chitosan of >100 kDa is less than that by chitosan of 15 and 52 kDa. Optimum molecular weight of chitosan is dependent on the cell lines. Chitosans of 10–50 kDa are excellent as gene transfer reagents. Molecular weight of chitosan may influence the stability of the DNA–chitosan complex, the efficiency of cell uptake, and the dissociation of DNA from the complex after endocytosis. In contrast, lipofectin displays a low luciferase activity owing to its low stability in serum. From the figure, it can be clearly seen that polygalactosamine (pGalN) does not show transfection efficiency at any pHs at all. In their previous work, Sato et al. observed that DNA–pGalN complex showed a sig-

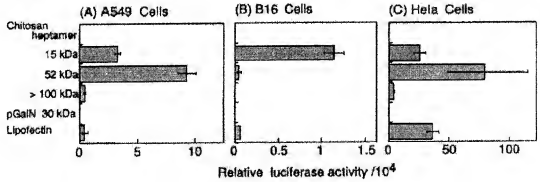


Fig. 2. Effect of various gene transfer reagents on the luciferase activity of (A) A549 cells, (B) B16 melanoma cells, and (C) Hela cells in DMEM (pH 6.9) containing 10% FBS. The ratios of pGL3:chitosan and pGL3:pGalN were 1:5. The weight ratio of plasmid:lipofectin was 1:2. Concentration of pGL3 was 10 μg/ml.

nificant uptake by blood cells and Hela cells. Obviously, the transfection efficiency does not correlate with the cell uptake. To achieve high transfection efficiency, plasmid must be ferried into nucleus and is capable of unpaking from its vector. Whereas, the experiment of pGL3–pGalN and pGL3–chitosan complex stability in 4% sodium dodecylsulfate (SDS) suggests that pGL3–pGalN complex is too stable to dissociate in endosome.

DNA delivered by non-viral carriers is vulnerable to degradation by DNase. Richardson et al. [49] reported that complexation of DNA with highly purified chitosan fractions (molecular weights of <5000, 5000–10 000 and $>10\,000$ Da) at a charge ratio of 1:1 resulted in almost complete inhibition of degradation by DNase II. All the above chitosan fractions displayed neither toxicity nor hemolytic activity, and low molecular weight chitosan can be administered intravenously without liver accumulation [48]. Nonetheless, it is worthwhile to note that the stability of complex in serum decreases when the molecular weight of chitosan is below 5000 [46,50].

It is found that at the serum content of 20%, gene expression in A549 cells with the pGL3–chitosan complex was increased about two to three times than that without serum. The increase of transfection efficiency in 20% serum is considered that the cell viability under this circumstance is optimal for transfection. Erbacher et al. has reported similar results [43]. When chitosan (average molecular weight, 245 kD)–salmon sperm DNA (average molecular weight, 236 kD) complex was incubated with Hela cells and blood monocytes, respectively, a significant result was obtained that the uptake efficiency for Hela cells was 44%; while the uptake efficiency by blood monocytes was merely 9%. Thereby, it was surmised that the DNA–chitosan complex could selectively transfect tumor cells in the low pH media without being uptaken into normal cells such as blood cells [51].

4. Chitosan derivative vectors

4.1. Deoxycholic acid modified-chitosan vector

Lee and Kim et al. [50,52] proposed to hydrophobically modify chitosan ($M_v=7.0\times10^4$, degree

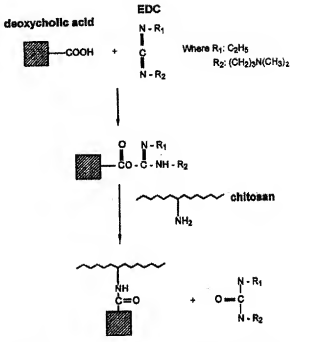


Fig. 3. A scheme of the coupling mechanism between chitosan and deoxycholic acid using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) through amide linkage formation.

of deacetylation = 80%) with deoxycholic acid (Fig. 3). Deoxycholic acid is a main component of bile acid, which is biologically the most detergent-like molecule in the body. Since bile acid can assemble in water, the deoxycholic acid-modified chitosan also self-associates to form micelles of a mean diameter of 160 nm.

Complex formation between chitosan self-aggregates and plasmid DNA was verified by electrophoresis of complexes on an agarose gel (Fig. 4). The analysis of retarding lanes can characterize the complex formation since the charge neutralization and increase in molecular size of the complexes hinder the migration of DNA on an agarose gel. As shown in the figure, above a charge ratio of 4/1 (lanes 7–11), the total retention occurs, i.e. the complex is formed.

The transfection of COS-1 cells (monkey kidney) with chitosan self-aggregate–DNA complexes was examined using the plasmid encoding chloramphenicol acetyltransferase (CAT). The transfection efficiency of the complexes is enhanced compared to

Fig. 4. Electrophoresis of deoxycholic acid-modified chitosan–DNA complexes on an agarose gel. Lane 1, DNA molecular weight marker II; lane 2, DNA only; lane 3, chitosan-DNA charge ratio=0.25/1; lane 4, 0.5/1; lane 5, 1/1; lane 6, 2/1; lane 7, 4/1; lane 8, 6/1; lane 9, 8/1; lane 10, 10/1; lane 11, 20/1.

that achieved by pristine DNA but lower than that achieved by Lipofectamine.

4.2. Dodecylated chitosan vector

An *N*-dodecylated chitosan (CS-12) was synthesized by us from dodecyl bromide and chitosan (average molecular weight, 700 kDa), and was assembled with DNA (salmon testes, average molecular weight, 2 kbp) to form a polyclectrolyte complex (DNA–CS-12 PEC) [53,54]. Incorporating dodecylated chitosan can enhance the thermal stability of DNA. Fig. 5 displays the atomic force microscopy (AFM) image of DNA–CS-12 complex. One can clearly see that DNA is aggregated and the complex appears globular in structure. The diameter varies form 9 to 230 nm, and the average height is about 18 nm. To approximately estimate the number of DNA molecule encapsulated in one globule, a rough quantitative analysis is made as follows: the total volume of the globule is calculated in terms of the volume of spherical cap:

$$V_G = \frac{1}{3}\pi h^2(3R - h) \tag{1}$$

where R and h are the diametcr and height of the spherical cap, respectively.

The total volume of DNA is

$$V_d = \pi r^2 L \tag{2}$$

where $r = 10$ Å. $L = n \times l$ where n is the number of base pairs, and l, the distance between two neighboring base-pair, is equal to 3.4 Å. The number of DNA molecules contained in one globule is

$$N = V_G / V_d \tag{3}$$

From the above formula, a single globule consists of 40~115 DNA molecules, which is shielded by chitosan, especially its long alkyl side chains.

Small molcular salts can dissociate the PEC, inducing DNA to release. The ability of Mg^{++} to dissociate PEC is greater compared to that of Na^{+} and K^{+}. Fig. 6 shows the AFM images of pure DNA and DNA releaseđ from complex with the addition of DNase. Pure DNA in the absence of dodecylated chitosan is hydrolyzed by DNase and has been broken into fragments. While DNA dissociated from the complex is well protected and remains intact due to the protection from DNase offered by alkylated chitosan. CS-12-mediated gene transfection is underway in our lab.

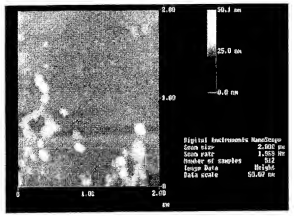
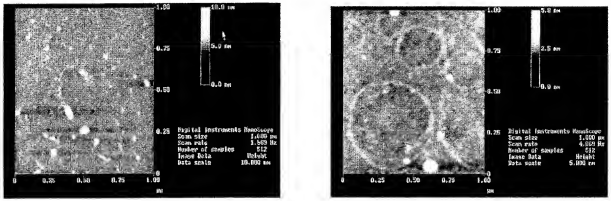


Fig. 5. AFM image of DNA–CS-12 complex. Chitosan–DNA charge ratio=2/1. The measurement is carried out in tapping mode. Reproduced with permission [53].

4.3. Quaternized chitosan vectors

Despite advantageous properties, chitosan is insoluble at physiological pH values. Whcreas only soluble protonated chitosan can cause the transient opening of cell membrane, promoting transmembrane transport of gene. To overcome this demerit, trimethyl chitosans (TMO) were prepared and examined for their potential as gene carrier in two cell lines, COS-1 and Caco-2 [55,56]. TMO with degrees



(A) (B)

Fig. 6. AFM images of pure DNA (A) and DNA released from complex (B) with the addition of DNase. A certain amount of DNA–CS-12 complex film and pure DNA were placed into deionized water, respectively, to which 10 μl of 200 μg/ml DNase solution was added. After 8 h of incubation at 37 °C, the complex film was washed repeatedly with deionized water and placed into 0.1 mol/l $MgCl_2$ solution for additional 48 h dissociation at 37 °C. Five microliters of pure DNA aqueous solution and salt solution of complex were deposited onto a freshly cleaved mica disk, respectively. The deposited solutions were dried at room temperature for AFM sample formation. The imaging was conducted in tapping mode. Reproduced with permission [53].

of quaternization of 40% (TMO-40) and 50% (TMO-50)–plasmid DNA complexes were tested for their ability to transfect COS-1 cells. In comparison, chitosan oligomers and DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium sulphate) liposomes were tested as well. The results are shown in Fig. 7.

As shown in the figure, DOTAP–DNA lipoplexes

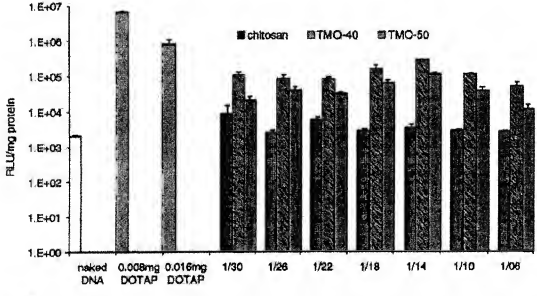


Fig. 7. Transfection efficiencies in COS-1 cells obtained with chitosan oligomer, trimethylated chitosan oligomers and DOTAP–DNA complexes at different weight/weight ratios. The RSV-α3 luciferase plasmid (5.6 kbp) was used to monitor gene transfer and transgene expression. Results are expressed in relative ligbt units (RLU) per mg protein. Values are mean ± S.D. ($n = 3$).

increase transfection efficiencies in COS-1 cells compared to the control group (naked DNA). Chitosan oligomer raises the transfection efficiency 2~4 times compared to the control values. TMO-50 markedly increases the transfection efficiencies from 5-fold (for complexes with DNA/oligomer ratio, 1/6) to 52-fold (for ratio 1/14). TMO-40 displays even higher transfection efficiencies ranging from 26-fold (for ratio 1/6) to 131-fold (for ratio 1/14). However, none of these vectors is able to remarkably increase the transfection efficiency in differentiated cells like Caco-2. Chitosan and trimethylated chitosan oligomers prove to be nontoxic on both types of cell in contrast to DOTAP that decreases viability to 50%.

4.4. Galactosylated chitosan vector

Although in some cases, uptake of chitosan–DNA nanoparticles appears to occur even in the absence of any ligand–receptor interaction [26], to allow for the targeted gene trafficking into specific cells, chitosan is required to be modified with various biospecific ligands. Various modification strategies can be used for this purpose including covalent attachment of the ligand moiety to the free amino groups of chitosan.

Recently, Park et al. [57] prepared galactosylated chitosan–graft–dextran–DNA complexes. Galactose groups were chemically bound to chitosan for liver-targeted delivery and dextran was grafted for enhancing the complex stability in water. This system could efficiently transfect Chang liver cells expressing asialoglycoprotein receptor (ASGR) which specifically recognizes the galactose ligands on chitosan. In subsequent work [58], they developed galactosylated chitosan–*graft*–PEG (GCP) vector. DNA–GCP complex was more stable due to hydrophilic PEG protection, and shielded from degradation by enzyme in plasma. Also, GCP has a small size (<100 nm) and GCP–DNA complexes were only transfected into HepG2 having ASGR, indicating galactosylated chitosan will be an effective hepatocyte-targeted gene carrier.

4.5. Transferrin–KNOB protein conjugated chitosan vector

Transferrin receptor responsible for iron import to the cells is found on many mammalian cells [59]. As a ligand, transferrin could efficiently transfer small molecular weight drugs, nonbioactive macromolecules, and liposomes through a receptor-mediated endocytosis mechanism [60]. In the past decade, transferrin has been applied to deliver plasmid DNA and oligonucleotides [61–63].

To improve transfection efficiency, Mao et al. [64] explored two strategies to bind transferrin onto the surface of chitosan–DNA complex.

The first is to produce aldehyde group to transferrin (a glycoprotein), by periodate oxidation. The modified transferrin was then reacted with the amino groups of chitosan (Fig. 8).

This scheme ensured that the modification only occurred at the polysaccharide chains of transferrin. In addition, a long spacer arm provided by polysaccharide chain in transferrin minimized the loss of the binding activity of transferrin. The transfection experiment was performed on human embryonic kidney 293 cells (HEK293) using pRE-luciferase as a model plasmid. The transferrin-conjugated nanoparticles with 14.2% modification degree yielded levels twice as high as unmodified ones; whereas 5.3 and 32.7% conjugation degree only generated similar levels. A decrease in transfection ability at high amount of transferrin is presumably due to the self-conjugation of itself.

In the second strategy, transferrin was linked to the nanoparticle surface through a disulfide bond

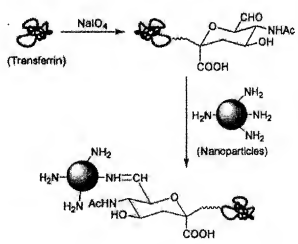


Fig. 8. Conjugation of transferrin through periodate oxidation.

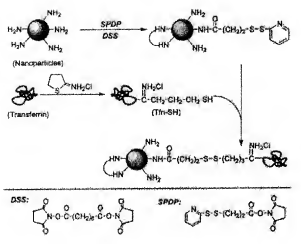


Fig. 9. Conjugation of transferrin through a reversible disulfide linkage.

(Fig. 9). The transferrin-conjugated vector only resulted in a maximum of 4-fold increase in transfection efficiency in HEK293 cells and only 50% increase in HeLa cells.

To further enhance transfection efficiency, KNOB (C-terminal globular domain of the fiber protein) was conjugated to the chitosan by the disulfide linkages as well. The KNOB conjugation to the nanoparticles could improve gene expression level in HeLa cells by 130-fold.

5. Conclusion remarks

The accumulated information about the physicochemical and biological properties of chitosan has led to the recognition of this cationic polysaccharide as a promising and versatile non-viral vector for gene transfection. By varying the molecular weight of chitosan, plasmid concentration, stoichiometry of polymer–plasmid complex, serum concentration, and pH of medium, the transfection efficiency and cell uptake can be tuned; a chemically modified chitosan, in particular the ligand-coupled can ferry gene into targeted cells. What's more, some encouraging results have been reported on in vivo administration routes of chitosan-based gene transfection system, such as oral, nasal and intravenous administration [49,65]. However, in our opinion, to gaining a deep insight into the transfection mechanism of chitosan-based non-viral vectors, several critical problems need to be addressed, for example, little is known about the transport of DNA into the nucleus, studies on the unpacking of DNA from chitosan vector remains insufficient. Last but not least, much more work is merited to develop targeted chitosan-based vector for specific tumor cells.

Acknowledgements

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